

## Matrix Metalloproteinases and Their Inhibitors: Correlation with Invasion and Metastasis in Oral Cancer

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**Abstract** Matrix metalloproteinases (MMPs) have been implicated in invasion and metastasis of various malignancies. The study evaluated a comprehensive profile of MMP-2 and MMP-9 and their inhibitors, tissue inhibitor of metalloproteinases-2 (TIMP-2) and tissue inhibitor of metalloproteinases-1 (TIMP-1), respectively in 50 controls and 75 patients with oral squamous cell carcinoma (OSCC). Blood samples from controls and patients as well as malignant and adjacent normal tissues from the patients were collected. The study examined pro, active and total forms of MMP-2 and MMP-9 using zymography. Enzyme-linked immunoassay (ELISA) and reverse transcription polymerase chain reaction were carried out to evaluate protein levels and mRNA expression; respectively, for the MMPs and TIMPs. Plasma pro, active and total MMP-2, MMP-9 as well as TIMP-1 and TIMP-2 levels were significantly higher in oral cancer patients as compared to the controls. mRNA expression of the MMPs and TIMPs was significantly higher in malignant tissues as compared to adjacent normal tissues. A significant positive correlation was observed between levels of proMMP-9 and active MMP-9 with differentiation, stage and infiltration. ProMMP-2 and active MMP-2 exhibited significant positive correlation with differentiation and lymph node involvement. The multivariate analysis of ELISA results revealed a significant positive correlation between MMP-2, TIMP-1

and TIMP-2 levels with lymph node involvement, stage and differentiation. The receiver operating characteristic curve (ROC) analysis showed that the levels of MMPs and TIMPs have significant discriminatory efficacy to differentiate between controls and patients. The results indicate that MMP-2, MMP-9, TIMP-1 and TIMP-2 have significant clinical usefulness for oral cancer patients. Zymographic analysis is a simple, cost effective, rapid and sensitive alternative assay.

**Keywords** MMPs · TIMPs · Oral cancer · Zymography · Metastasis · Invasion

### Introduction

Globally, oral cancer is a major health hazard, with 270,000 new oral cancer cases and 145,000 deaths annually, of which two-thirds occur in developing countries [1, 2]. The highest incidence of oral cancer has been observed in the Indian subcontinent [3].

Oral cancer is characterized by a high degree of local invasiveness and a high rate of metastasis to cervical lymph nodes. Death as a result of cancer is often the result of local recurrence or regional and/or systemic metastasis. Thus, metastasis is the strenuous problem in successful cancer treatment, and it is believed that they begin in the growth of the primary tumor [4]. Tissue invasion and metastasis require an extensive remodeling and degradation of extracellular matrix (ECM) which requires the concerted action of a number of extracellular enzymes [5]. Matrix metalloproteinases (MMPs) belong to the group of ECM degrading enzymes. The balance of these enzymes and their specific inhibitors play an important role in maintaining connective tissue homeostasis [6]. Imbalances in

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the extra cellular activities of MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs) have been linked with pathological destruction in cancer. The over-expression of gelatinases and their tissue inhibitors has been shown to correlate with grade or stage of tumors in several different types of cancer [7]. Smith et al. [8] have reviewed the importance of MMP-2 and MMP-9 as markers for increased risk of progression from oral dysplasia to cancer. Considering oral malignancy as one of the leading malignancy in India, better markers for the prediction of cancer progression is therefore needed.

Evidences suggest that MMPs and their physiological tissue inhibitors (TIMPs) play a causal role in oral cancer progression [9]. Earlier reports on in vivo involvement of MMPs and TIMPs in oral cancer are incongruous and also their correlation with clinico-pathological variables is still contradictory, partly because of varying methods used for their detection. Also, there are very few studies in literature that have evaluated the combined role of MMPs and their inhibitors in tissue invasion and metastasis.

Because the components of the ECM are complex, the combined action of various MMPs is essential for the efficient degradation of the structure of the ECM. MMP-2 and MMP-9 and their physiological inhibitors (TIMP-2 and TIMP-1) were selected for the study because of the unique ability of MMP-2 and MMP-9 to degrade the type IV collagen, a major component of basement membrane. Degradation of type IV collagen is the prerequisite for tumor invasion. Hence, the aim of this investigation was to perform a comprehensive analysis of MMP-2, MMP-9, TIMP-2 and TIMP-1 in oral cancer patients using substrate zymography, enzyme-linked immunoassay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) and to find clinicopathological significance of these subset of enzymes.

## Materials and Methods

### Study Subjects

The study was approved by Scientific Review Committee and Ethics Committee of The Gujarat Cancer and Research Institute, Ahmedabad.

### Patients

A total of 75 untreated oral cancer patients were enrolled for the study. There were 55 men and 20 women, ranging in age from 25 to 70 years (mean, 43 years for males and 51 years for females). 49.3% patients had advanced disease. 28% showed lymph node metastasis. 37.3% had infiltrating tumor growth (Table 1).

**Table 1** Clinical details of oral cancer patients

Characteristics	N = 75
No. of patients	
Sex, N (%)	
Male	55 (73.3)
Female	20 (26.6)
Male:female	2.7:1
Age (years)	
Male—mean (range)	43 (25–70)
Female—mean (range)	51 (30–70)
Male:female (range)	0.84 (25–70)
Histopathology	Squamous cell carcinoma
Site, N (%)	
Buccal mucosa	32 (42.6)
Tongue	19 (25.3)
Alveolus	13 (17.3)
Others	11 (14.6)
Tumor differentiation, N (%)	
Well	20 (26.7)
Moderate	30 (40.0)
Poor	12 (16.0)
Undefined	13 (17.3)
Early (stage I + stage II) N (%)	28 (37.3)
Advanced (stage III + stage IV) N (%)	37 (49.3)
Not available	10 (13.3)
Lymph node involvement, N (%)	
Positive lymph node	21 (28)
Negative lymph node	54 (72)
Mode of invasion, N (%)	
Localized	47 (62.7)
Infiltrating	28 (37.3)

### Control Group

The control group consisted of 50 healthy volunteers from the blood bank of the institute as well as relatives of the oral cancer patients without any history of cancer, inflammation or any other major illness. There were 38 men and 12 women, ranging in age from 22 to 48 years (mean, 21 years for males and 33 years for females).

### Sample Collection and Processing

**Blood Samples** Blood samples from the subjects were collected by vein puncture in heparinized vacuettes. Plasma were separated and stored at  $-80^{\circ}\text{C}$  until analysis.

**Tissue Samples** A total of 42 tissue samples (21-paired tissues of malignant and adjacent normal tissues) were collected from oral cancer patients at the time of surgery from operation theatre. The tissues were histologically

examined and defined by pathologist as malignant and adjacent normal tissue (dissected from free margins at least 1 cm away from tumor). The tissue samples were collected on ice, washed with chilled phosphate buffered saline (PBS) (pH 7.4). All the samples were stored at –80°C until analysis.

#### Preparation of Euglobulins

Euglobulin fractions were prepared as described by Ranuncolo et al. [10] with minor modifications. Briefly, 0.1 ml plasma sample was mixed with 0.9 ml chilled deionised distilled water and acidified with 40 µl of 1% acetic acid and incubated at 0°C for 1 h. Then, centrifuged at 5,000 rpm for 10 min. Euglobulin fraction (as pellet) was collected and dissolved in PBS.

#### Zymography

Zymography was performed using SDS-PAGE (containing 0.5 mg/ml gelatin) electrophoresis as described by Lorenzo et al. [11]. The euglobulin fraction was mixed with sample buffer dye without reducing agent and electrophoresed on 7.5% polyacrylamide gel at 4°C. Gels were washed and incubated overnight in 50 mM Tris HCl pH 7.5; containing 10 mM CaCl<sub>2</sub>, 1 µM ZnCl<sub>2</sub>, 0.02% NaN<sub>3</sub> and 1%(v/v) Triton X-100. Next day, the gels were stained with 0.1% Coomassie Brilliant Blue-R-250 (v/v) and destained in 7% acetic acid. These zymograms were quantitated using gel documentation system (Bio-Rad G-800). The relative proteinase activity was determined for each proteinase by multiplying the area of each lysed band by its optical

density (OD\*mm<sup>2</sup>). Commercially available pure human MMP-2 and MMP-9 (Calbiochem, San Diego, CA) were used as standards.

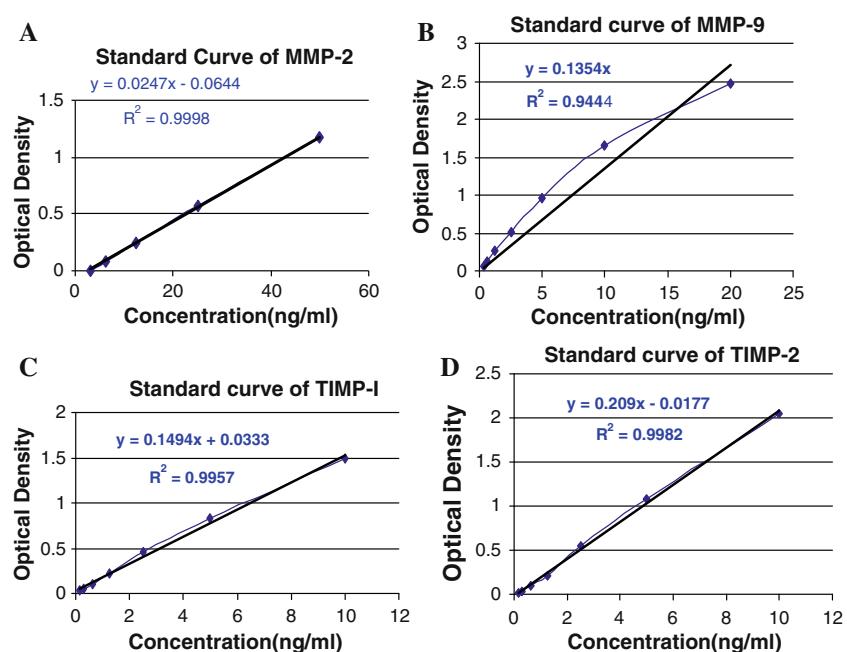
#### Enzyme-Linked Immunoassay

Concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the same plasma samples as used in zymography were measured using commercially available ELISA kits (R&D system, Inc., USA). The ELISA were carried out as per manufacturer's instructions. ELISA values were measured in duplicate for each sample in order to minimize intra-assay variation. The absorbance values for standard samples and the standard curves (Fig. 1) constructed for each assay were compared and used to minimize the inter-assay variation. MMP-2, MMP-9, TIMP-1 and TIMP-2 concentration in the samples were determined by comparing the optical density of the samples to the standard curves. The intra-assay variability for MMP-2, MMP-9, TIMP-1 and TIMP-2 was 5.6, 5.2, 5.0, 5.9% respectively and the inter-assay variability for MMP-2, MMP-9, TIMP-1 and TIMP-2 was 6.2, 5.9, 6.0 and 5.5% respectively.

#### Reverse Transcription Polymerase Chain Reaction

Total ribonucleic acid (RNA) was isolated from tissue samples obtained from oral cancer patients using RNeasy Mini Kit (Qiagen, USA) according to manufacturer's instructions. RNA was suspended in RNAase free water and stored at –80°C until use. The following genes were assayed using specific primer pairs: MMP-2, MMP-9, TIMP-1, and TIMP-2. Glyceraldehyde-3-phosphate

**Fig. 1** Standard curve of  
a gelatinase-A (MMP-2),  
b gelatinase-B (MMP-9),  
c TIMP-1 and d TIMP-2



**Table 2** Primers used for RT-PCR analysis

Target gene	Primer sequences (5'-3')	Target accession number	Position	Product size (bp)
MMP-2	GGC CCT GTC ACT CCT GAG AT	J03210	1337–1356	474
	GGC ATC CAG GTT ATC GGG GA		1810–1791	
MMP-9	GGC CCT GTC ACT CCT GAG AT	NM004994.1	1554–1573	455
	GGC ATC CAG GTT ATC GGG GA		2008–1989	
TIMP-1	AGC GCC CAG AGA GAC ACC	X03124.1	33–50	670
	CCA CTC CGG GCA GGA TT		702–686	
TIMP-2	GGC GTT TTG CAA TGC AGA TGT AG	NM003255.1	378–400	497
	CAC AGG AGC CGT CAC TTC TCT TG		874–852	

dehydrogenase (GAPDH) gene was used as an internal control. The primer sequences are mentioned in Table 2.

### Statistical Analysis

The data were statistically analyzed using SPSS statistical software [version 10.0]. Student's *t* test was used to compare mean levels of the parameters between various groups of the subjects. Receiver's operating characteristic (ROC) curves were constructed with the program MedCalc version 8.2.0.1 (MedCalc Software, Mariakerke, Belgium) to determine the efficacy of the markers to discriminate between various groups of the subjects. The best statistical "cut-off" was calculated by minimizing the distance between the point with specificity = 1 and sensitivity = 1 and the points on the ROC curve. The areas under curve (AUC) were reported with their 95% confidence intervals. Multivariate analysis was carried out to correlate markers with clinicopathological parameters. Relative comparison of bands obtained through the RT-PCR between adjacent normal and malignant tissues of oral cancer patients was carried out by densitometry analysis as OD\*mm<sup>2</sup>. Values were expressed as mean ± SEM. *P* ≤ 0.05 were considered statistically significant.

## Results

### Expression of MMPs and TIMPs in Oral Cancer

#### Quantitation of MMP Activity by Substrate Zymography

We used substrate zymography to determine the activity of MMP-2 and MMP-9 from plasma euglobulin fraction. Gelatin zymography revealed a varied profile of MMPs. The lysis zones corresponding to molecular weights of 92, 84, 72 and 62 kD gelatinases were seen (Fig. 2). Gelatinases of 92 and 84 kD were corresponding to proMMP-9 and an active form of MMP-9, respectively. Those of 72

and 62 kD were considered to be proMMP-2 and its active form respectively.

The computerized image analysis of transparent bands, revealed significantly higher (*P* = 0.001) mean levels of proMMP-2, active MMP-2, total MMP-2, proMMP-9, active MMP-9, total MMP-9 activities in oral cancer patients as compared to the controls (Fig. 3).

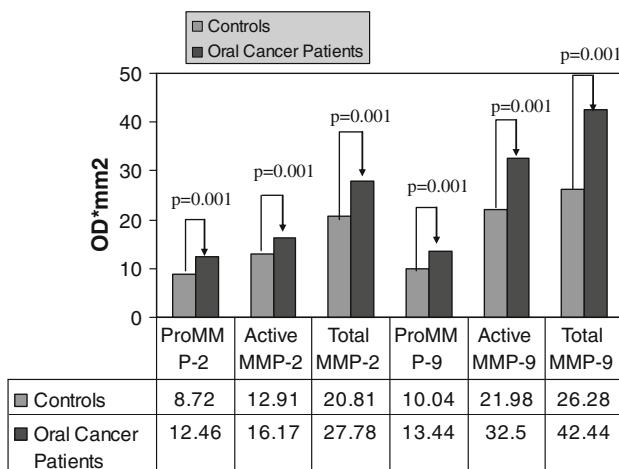
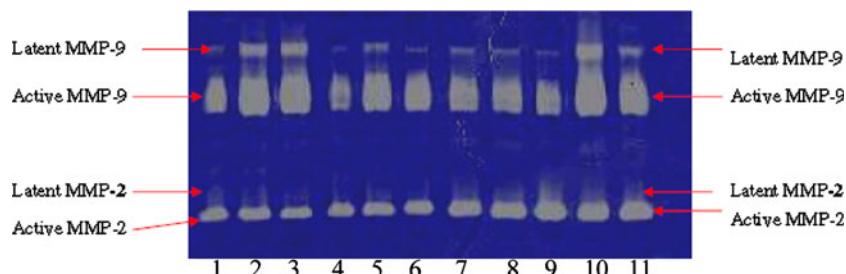
**ROC Curve Analysis of Different Forms of MMP-2 and MMP-9 Obtained by Substrate Zymography** The ROC curve revealed that the mean levels of proMMP-2, active MMP-2, total MMP-2, proMMP-9, active MMP-9 and total MMP-9 could significantly distinguish between oral cancer patients and controls (Fig. 4). The area under the curve for pro-MMP-2, pro-MMP-9, active MMP-2, active MMP-9, total MMP-2 and total MMP-9 was 0.81, 0.78, 0.77, 0.88, 0.83 and 0.90, respectively.

#### Quantitation of MMPs and TIMPs by ELISA

Table 3 depicts comparison of MMP-2, MMP-9, TIMP-1 and TIMP-2 levels in plasma obtained by sandwich ELISA between controls and oral cancer patients. MMP-2 levels were elevated in oral cancer patients as compared to the controls. Mean values of MMP-9, TIMP-1, TIMP-2 levels were significantly higher (*P* = 0.001, 0.012, 0.025) in oral cancer patients as compared to the controls.

**ROC Curve Analysis of MMP-2, MMP-9, TIMP-1 and TIMP-2 Levels Obtained by Sandwich ELISA** Plasma MMP-9, TIMP-1 and TIMP-2 could significantly discriminate (*P* = 0.001, 0.027, 0.022, respectively) between controls and oral cancer patients. The AUC for plasma MMP-2, MMP-9, TIMP-1 and TIMP-2 were 0.60, 0.83, 0.67 and 0.68, respectively (Fig. 5). The ROC analysis showed an AUC of 0.83 for MMP-9 with the best statistical cut-off of 226.29 ng/ml (sensitivity = 68.1%; specificity = 95.2%). The AUC for MMP-2 was 0.60 with a best statistical cut-off of 167.98 ng/ml (sensitivity = 57.4%; specificity = 71.4%). The AUC for

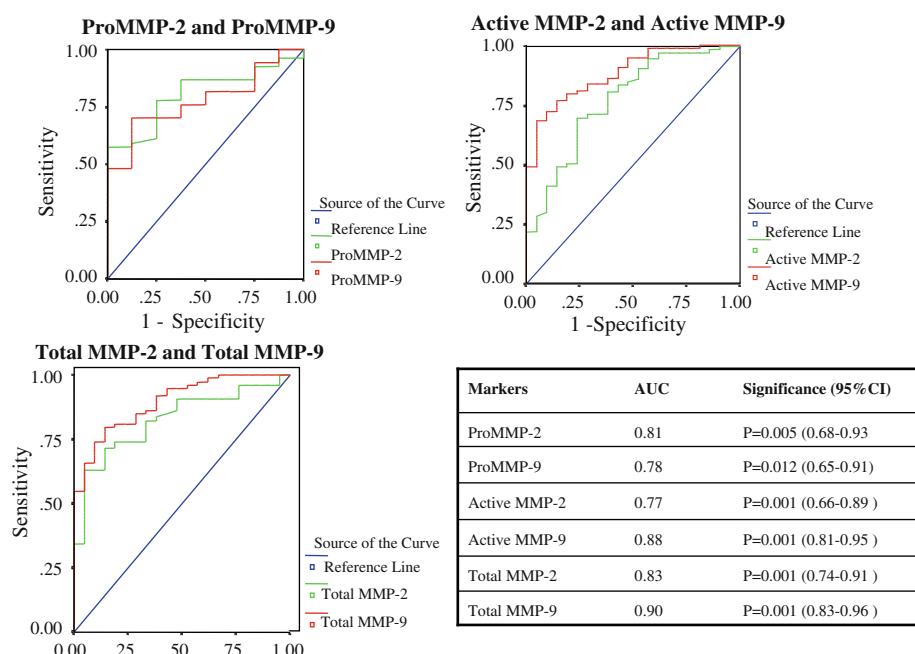
**Fig. 2** Representative gelatin zymogram of plasma euglobulin fraction from oral cancer patients. Lanes 1, 2, 3, 5, 10, 11 patients with carcinoma buccal mucosa. Lanes 4, 6, 7, 8, 9 patients with carcinoma tongue



**Fig. 3** Comparison of pro, active and total forms of MMP-2 and MMP-9 between controls and oral cancer patients

TIMP-1 was 0.67 with a cut-off greater than 65.16 ng/ml (sensitivity = 74.5%; specificity = 61.9%). For TIMP-2, AUC was 0.68 with a cut-off greater than 95.38 ng/ml having sensitivity = 52.08% and specificity = 90.0%.

**Fig. 4** ROC curve for pro MMP-2, proMMP-9, active MMP-2, active MMP-9, total MMP-2, total MMP-9 between oral cancer patients and controls for the results obtained by zymography



**Table 3** Comparison of mean levels (ng/ml) of plasma MMP-2, MMP-9, TIMP-1, TIMP-2 obtained by Sandwich ELISA between controls and oral cancer patients

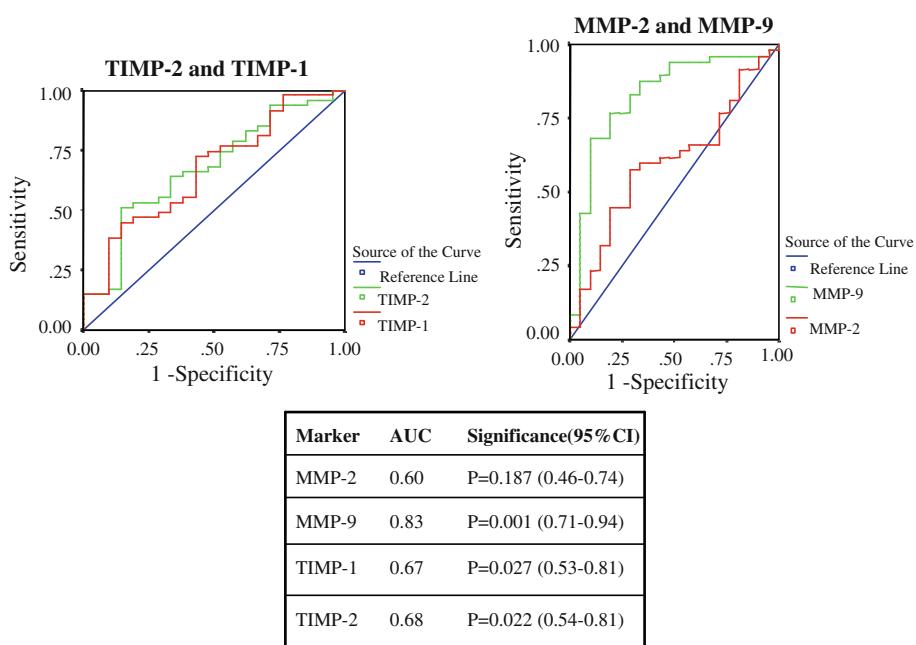
Parameters	Control (mean ± SEM)	Oral cancer patients (mean ± SEM)
Plasma MMP-2	161.97 ± 9.10	180.84 ± 7.57
Plasma MMP-9	148.64 ± 29.87	352.30 ± 36.55*
Plasma TIMP-1	63.37 ± 5.06	84.73 ± 6.60*
Plasma TIMP-2	87.54 ± 3.61	98.56 ± 3.10*

\* Statistically significant compared to control ( $P < 0.05$ )

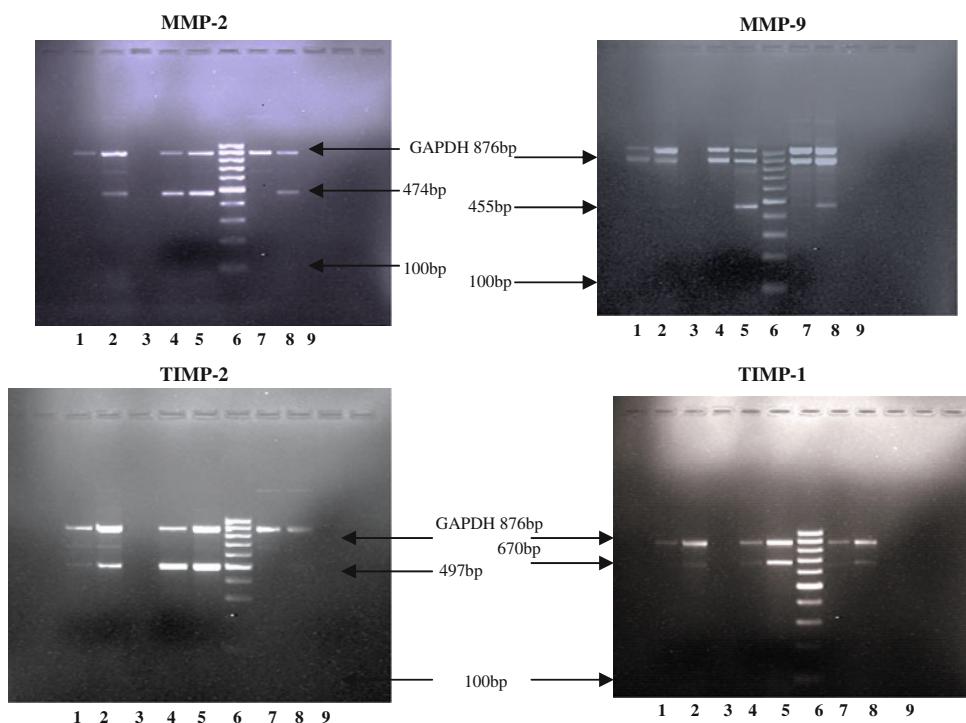
#### MMP-2, MMP-9, TIMP-2 and TIMP-1 mRNA Expression

Histologically normal adjacent mucosa and primary malignant tissue from the same patients were examined for mRNA levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 (Fig. 6). GAPDH (876 bp) gene was used as internal control in all the RT-PCR experiments. Using the RT-PCR assay, mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2 showed 1.8-fold, fourfold, 2.4-fold and 1.3-fold

**Fig. 5** ROC curve for MMP-2, MMP-9, TIMP-1 and TIMP-2 between oral cancer patients and healthy controls for the results obtained by ELISA



**Fig. 6** Representative pattern for mRNA expression of MMP-2, MMP-9, TIMP-2 and TIMP-1 by RT-PCR. Lanes 1, 4, 7 adjacent normal tissues; Lanes 2, 5, 8 malignant tissues; Lane 6 molecular weight marker; Lane 3, 9 negative control

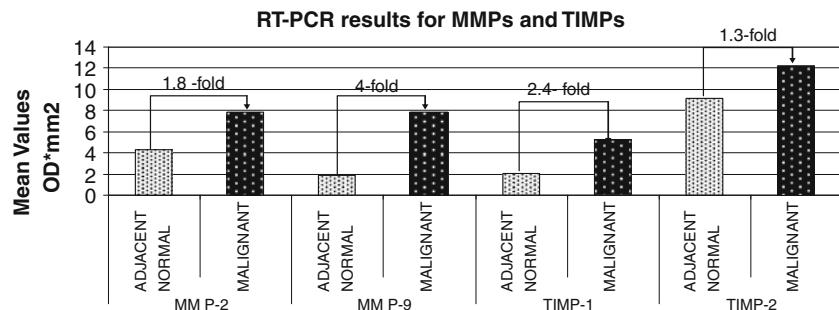


increase in malignant tissue (primary tumors) as compared with mRNA levels in adjacent normal mucosa (Fig. 7).

Relationship between the levels of activity of MMPs and TIMPs in oral cancer patients and their clinicopathological variables were analyzed by multivariate analysis. As documented in Table 4 proMMP-9 activity in primary squamous cell carcinoma (SCC) showed a statistically significant relationship with differentiation ( $P = 0.008$ ). A significant positive correlation was also found between

proMMP-9 activity and stage of the tumor ( $P = 0.040$ ). Advanced stage of the tumor showed significantly higher proMMP-9 activity in comparison to the early stage ( $P = 0.051$ ). Active MMP-9 showed statistically significant correlation ( $P = 0.014$ ) with infiltrating pattern of growth of tumor. Pro MMP-9 and active MMP-9 levels were weakly correlated with the presence of lymph node involvement ( $P = 0.070, 0.061$ ) respectively. Pro MMP-2 and active MMP-2 showed a strong correlation with degree

**Fig. 7** Comparison of mean values of mRNA expression of MMP-2, MMP-9, TIMP-2 and TIMP-1 in malignant tissue and adjacent normal mucosa of oral cancer patients



**Table 4** Levels of MMPs and TIMPs in relation to clinicopathological variables

Clinicopathological variable	STAGE		EARLY_ADVANCE		LYMPH NODE INVOLVEMENT		INFILTRATING_LOCALIZED		DIFFERENTIATION	
	F	Significance	F	Significance	F	Significance	F	Significance	F	Significance
Pro-MMP-9 <sup>a</sup>	4.76	0.040	4.23	0.051	3.60	0.070	1.29	0.268	8.31	0.008
Active MMP-9 <sup>a</sup>	2.38	0.137	0.82	0.375	3.86	0.062	7.00	0.014	2.22	0.150
Total MMP-9 <sup>a</sup>	2.85	0.105	2.97	0.098	3.34	0.081	2.94	0.100	3.84	0.062
Pro-MMP-2 <sup>a</sup>	3.75	0.065	2.97	0.098	0.72	0.406	2.30	0.143	5.69	0.026
Active MMP-2 <sup>a</sup>	0.65	0.428	1.14	0.297	4.27	0.050	1.57	0.223	0.66	0.426
Total MMP-2 <sup>a</sup>	1.98	0.173	2.36	0.138	4.04	0.056	2.50	0.122	2.51	0.127
MMP-2 <sup>b</sup>	3.80	0.066	1.45	0.238	4.26	0.053	1.60	0.222	0.13	0.721
MMP-9 <sup>b</sup>	0.60	0.450	0.003	0.960	0.00	0.993	0.74	0.401	0.06	0.813
TIMP-1 <sup>b</sup>	1.11	0.317	3.24	0.088	3.97	0.061	0.17	0.687	3.74	0.068
TIMP-2 <sup>b</sup>	0.10	0.781	0.32	0.579	4.15	0.056	0.77	0.390	0.40	0.534
MMP-2/TIMP-2 <sup>b</sup>	3.22	0.088	0.77	0.392	0.15	0.705	0.29	0.596	0.04	0.848
MMP-9/TIMP-1 <sup>b</sup>	0.001	0.970	0.77	0.393	0.74	0.399	1.83	0.191	0.09	0.772

Note: <sup>a</sup> Results by zymography; <sup>b</sup> Results by ELISA

of differentiation and lymph node involvement respectively ( $P = 0.026, 0.050$ ). When the results obtained by ELISA for MMPs and TIMPs were subjected to multivariate analysis, a significant correlation was found between MMP-2 levels and lymph node involvement ( $P = 0.053$ ) and a weak correlation was observed between MMP-2 and stage of the tumor ( $P = 0.066$ ), TIMP-1 and lymph node involvement and degree of differentiation ( $P = 0.061, 0.068$ ), TIMP-2 and lymph node involvement ( $P = 0.056$ ). However, no association was observed between ratio of MMP-2/TIMP-2 and MMP-9/TIMP-1 and clinicopathological variables.

## Discussion

The metastatic spread of oral cancer is a major clinical problem and is responsible for a majority of cancer related deaths, due to tumor involvement of critical organs or to complications in therapies aimed at controlling tumor growth [12]. Proteolytic degradation of ECM is an essential part of this process and several enzyme systems like serine

proteinases, cysteine proteinases and MMPs have been shown to be involved [13]. The first step in metastasis formation involves the degradation of the underlying basement membrane which mainly consists of type IV collagen [14]. MMP-2 and MMP-9 play an important role in its degradation because of their ability to destroy this type of collagen [15].

Several studies have shown that gelatinases and their tissue inhibitors are over-expressed in head and neck carcinoma cells, and play a crucial role in progression and invasion of these tumors [16–18]. Previous reports have suggested a strong association between gelatinase mRNA, immunoreactive protein or enzyme activity and invasion or lymph node metastasis in head and neck cancer [19–21]. According to immunohistochemical staining results, 52–83 and 34–100% of head and neck cancer have been positive for MMP-9 and MMP-2 immunoreactive protein respectively [4]. Ruokolainen et al. [15] reported over-expression of MMP-9 immunoreactive protein in 82% patients of head and neck cancer but the authors failed to find any association between MMP-9 protein expression with stage and grade of the tumor. However, Vicente et al. [4] were able to

demonstrate over-expression of MMP-9, MMP-2 in 17.6% and 32% of oral SCC (OSCC) patients, respectively. They also showed highly significant correlation of MMP-2 and MMP-9 with lymph node metastasis. Ikebe et al. [20] showed that high levels of MMP-2 and MMP-9 were related to the invasiveness of oral SCC, whereas Charous et al. [18] showed no difference in MMP-2 and MMP-9 levels between primary head and neck cancer and lymph node metastasis.

It is clearly evident that there is a wide range of variations for the results for MMP-2 and MMP-9 expression and their correlation with invasion and metastasis obtained through immunohistochemistry. It can be attributed to use of different antibodies and heterogeneity of oral cancer. So far, the attempts to correlate gelatinase expression with clinical outcome for patients with head and neck cancer have been inconclusive and the predictive value of the MMPs and TIMPs in invasion and metastasis of head and neck cancer has been controversial. This may be partly because of the different methodologies used to detect MMP expression and partly because of the contradictory facts related to role of TIMPs on tumorigenesis and also because there is dearth of data in the literature showing combined analysis of MMPs and TIMPs in oral cancer. Overall and Kleifeld [22] introduced the term “protease web” and underlined that interaction between MMPs and TIMPs and related components is more important than the single component. This suggests that MMPs and TIMPs should be analyzed together in same set of patients to obtain more conclusive results.

We quantified the activity and expression of MMP-2, MMP-9, TIMP-2 and TIMP-1 using various methods including, substrate zymography, ELISA in blood plasma from patients with primary oral cancer at different stages and with or without metastasis taking into the account that MMPs are synthesized in tissues and released into the blood stream. We also performed highly sensitive RT-PCR assays for the mRNA expression of MMP-2, MMP-9, TIMP-2 and TIMP-1. The relationship of MMP-2, MMP-9, TIMP-2 and TIMP-1 with clinicopathological variables was also analyzed, in an attempt to determine whether over-expression of certain specific proteases could be particularly relevant to invasion and metastasis of this disease.

The results of the current study showed significantly increased circulating MMP-9 levels in patients as compared to their normal counterparts. These results are in agreement with the results of previous studies [10, 23, 24]. The zymographic analysis showed significant correlation for proMMP-9 with stage and degree of differentiation of the tumor. Also, active MMP-9 detected by zymography significantly associated with infiltrating pattern of tumor growth. However, MMP-9 failed to show any association with lymph node involvement. Like MMP-9, MMP-2 was also significantly elevated in oral cancer when analyzed

through zymography and ELISA which are in line with the results obtained by Sutinen et al. [25]. When the results of the present study for MMP-2 activity and expression obtained by both the techniques were subjected to multivariate analysis, MMP-2 showed positive association with lymph node involvement. These results are in agreement with the studies by Hong et al. [26], Tokumaru et al. [27], Miyajama et al. [21] and Kawamata et al. [17] who have concluded that MMP-2 were closely related to lymph node metastasis. However, we did not reach to statistical significance. This can be due to the complexity of the metastatic process which involves multiple MMPs and TIMPs. An interesting observation that the current study demonstrated was that the expression of proMMP-2 and proMMP-9 significantly associated with the degree of differentiation of the tumors.

We also analyzed expression of MMP-2 and MMP-9 using RT-PCR assay. There was around twofold and fourfold increase in amount of MMP-2 and MMP-9 in primary oral cancer tissue's expression over histologically normal adjacent tissues respectively. These results are similar with the study from O-charoenrat et al. [9]. Our results from the tissue confirm that circulating levels of MMPs and TIMPs are reflecting the direct tissue situation. Also significantly increased levels of latent, active and total forms of MMP-2 and MMP-9 strengthen results we have obtained in our lab in an earlier study in oral cancer and breast cancer with tissue as specimens [28, 29].

In the process of a tumor's invasion and metastasis, the secretion and activation of metalloproteinases are not enough to ensure that they will degrade the target matrix substrate. This is because that the natural TIMPs can block MMPs. A balance between MMPs and TIMPs is therefore essential. There is dearth of the data in the literature regarding TIMP expression in oral cancer. In the present study, we found increased levels of TIMP-1 and TIMP-2 proteins in plasma of oral cancer patients as well as primary tissue also showed 2.4-fold and 1.3-fold increase in TIMP-1 and TIMP-2 mRNA expression levels in comparison to histologically adjacent normal mucosa respectively. The findings of increased TIMP-1 and TIMP-2 expression in oral cancer might be explained by the growth-promoting activity of TIMPs on a variety of cell types [9] or the induction of TIMPs by secreted MMPs (or vice versa) from tumor-host interaction in the extracellular milieu. Few studies have also tried to analyze TIMP expression with clinicopathological variables. Although inhibition of *in vitro* and *in vivo* tumor invasion by TIMPs has been demonstrated, [30], increased, rather than decreased, TIMP levels have been shown to be related to poor outcome in several malignant tumors [9]. TIMP-1 over-expression has been shown to correlate positively with pattern of invasion [31]. TIMP-2 over-expression has been linked with local

tumor invasion, nodal status and clinical stage as well as with disease-free survival in oral cancer, especially in tongue SCC [31, 32]. However, O-charoenrat et al. [9] failed to find a correlation between TIMP-2 over-expression and clinicopathological factors. But with the results of the present study we could demonstrate that TIMP-1 and TIMP-2 protein levels are closely correlated with lymph node metastasis and degree of differentiation.

To the best of our knowledge this is the first study from India, in which combined activity and expression of gelatinases and their tissue inhibitors was explored in plasma and tumor tissue specimens of OSCC patients simultaneously. The use of gelatinases and their tissue inhibitors as clinical markers indicating the aggressiveness and metastatic potential of the oral cancer seems to be both very inviting and reasonable. However, still the question remains: what is the most accurate marker in each individual cancer type, and which laboratory method is the most accurate to use? The ROC curve analysis in the current study shows that pro MMP-2, active MMP-2, total MMP-2, proMMP-9, active MMP-9, total MMP-9 could significantly discriminate between controls and OSCC patients. Total MMP-9 documented highest discriminatory efficacy among them which is in accordance with the data reported by Ranuncolo et al. [10]. This finding is also supported by our results obtained through ELISA. These findings also strengthens the fact that zymographic analysis has advantages over immunologic assays, due to lower cost, rapid time of execution and the possibility of simultaneously detecting multiple forms of the same enzymes. Reproducibility and cost effectiveness of gelatin zymography makes it possible for routine analysis for each patient.

To sum up, the biology of aggressiveness of oral cancer is reflected into its ability to metastasize to regional lymph nodes. Monitoring the activity and expression levels of gelatinases and TIMPs in plasma of patients with cancer is a fascinating possibility. The approach using blood-based parameters has advantage of noninvasive method, sample availability during post-treatment follow-ups and possibility of repeat sampling. Zymographic tools have advantages over immunologic assays in terms of cost effectiveness, and above all identification of all the forms of gelatinases by this technique which has significant clinical utility in invasion and metastasis. Thus, the present findings documented significant clinical usefulness of the MMPs and TIMPs.

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